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Biocatalytic acylation of glucose by immobilized *Ricinus Communis* lipase

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Abstract

The aim of the present study is the enzymatic synthesis of sugar ester catalyzed by an immobilized lipase from the seeds of castor oil plant (R*icinus communis*). Experiments were focused on the synthesis of a glucose ester by selecting different acyl donors in different organic solvents. The highest esterification yield was obtained with palmitic acid in tert-Butanol. Effects of different parameters (temperature, water activity, glucose/palmitic acid molar ratio and immobilized lipase activity) on the synthesis of glucose palmitate were studied by varying one factor at a time. Maximum conversion yield (65%) was obtained under the optimized conditions: temperature (40 °C), glucose/palmitic acid molar ratio (1:2), water activity (0.3), stirring rate (600 rpm), lipase activity 2.7 U and reaction time (8 h). The structural characteristics of glucose palmitate were confirmed by FT-IR, ^{1}H and ^{13}C- NMR analysis. The glucose palmitate could reduce the surface tension of water from 72 to 42 mN/m with a CMC of 0.1 g/L.

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1. Introduction

Glucose esters or glucose fatty acid esters are the ester of glucose with fatty acids. Formation of the ester bond is called acylation. Glucose esters are non-ionic surfactant which can be used in many areas, pharmaceutical, cosmetic, detergents, agriculturel and food industries (**Thanongsak, 2007; Yu, 2008**). They are molecules which have an amphiphilic character. This feature gives them a special affinity for interfaces of air / water and oil / water type and, consequently, the ability to lower the free energy of these interfaces. This phenomenon is the basis for the stabilization of dispersed systems (**Salvator *et al*., 2001)**. The use of lipase catalysis makes regioselective acylation possible under mild conditions. **(Adlercreutz, 2013)**. Further, use of immobilized enzyme simplifies the downstream processing.

This study will focus on lipase of vegetal origin; they are present in the plant well that found mainly in seeds. From an industrial point of view, the interest of vegetal lipases has continued to increase in recent years, particularly in the field of lipid metabolism. Indeed, plant lipases are easily isolated from seeds and they exhibit unique substrate specificities compared to microbial lipases **(Beisson F., 2001).** In the present paper, we were interested in the synthesis of a glucose ester as a product of an esterification reaction between glucose and an acyl donor in the presence of *Ricinus communis* immobilized lipase. Aiming to maximize acylation yield, the effects of various parameters such as the nature of organic solvent, acyl donor, molar ratio of reactants and enzyme activity were studied in order to determine the optimal conditions. The structure of the glucose ester was analyzed by FTIR, ^{1}H ^{13}C- NMR and the critical micellar concentration was estimated.

2. Materials and methods

2.1. ***Chemicals***

Acetone (essay ≥ 99.9%) was purchased from Sigma-Aldrich. α-D-Glucose anhydrous, (96%), chloroform (≥ 99%), Stearic acid (95%) and oleic acid (90%) were from Riedel-de Haën. Ethyl acetate (99.8%) and methanol (99.7%) were from PA (Panreac). Tert-butanol (99%) and Palmitic acid (97% (GC)) were from Fluka.

2.2. ***Extraction of lipase***

Lipase (E.C. 3.1.1.3) was extracted from castor seeds (*Ricinus communis*) by a simple method. The seeds were shelled and crushed in a mortar with the addition of distilled water. The crude enzyme extract was then filtered through four layers of cheese cloth. Partial purification of lipase crude extract was carried out by acetone precipitation (**Sammour, 2005*;* Beisson *et al,* 2000; Ejedegba *et al,* 2007**). All operations of isolation of enzyme were performed at 4 °C. Recovery of semi-purified lipase was carried out by centrifugation at 3000 rpm for 5 minutes in a Remi R-24 cooled centrifuge (Remi Laboratory Instruments, Mumbai, India). The recovered pellet was dried at room temperature and then stored in a closed container at 4°C until use.

2.3. ***Lipase immobilisation***

The immobilization of *Ricinus communis* lipase was performed at room temperature. Partially purified lipase (0.5-2 g) was dissolved in 10 ml of distilled water. Sodium alginate (0.2 g) was then added to the enzyme
solution and the mixture was kept under magnetic agitation for one hour till a homogeneous mixture was obtained. Calcium alginate beads were prepared by extrusion using a simple one-step process (**Keehoon *et al,* 2005**). The entrapped TP beads were prepared by pouring the mixture drop wise using a syringe needle (20 G) in 50 mL of a solution of $CaCl_2$ concentration (0.05 M) under stirring. Beads were left in the $CaCl_2$ solution for 1 hour, then washed with distilled water and stored in a buffer solution (pH 7.0) in a refrigerator (4 °C). Immobilized lipase was contained

in spherical particles, with an average diameter of 3.0 mm were obtained. Immobilized lipase activity was estimated by titrimetric method (**ELISA D. et *al*, 2007**)

2.4. Enzymatic esterification reaction in organic solvent

Glucose ester was synthesized by acylation of glucose (acyl acceptor) using fatty acids (palmitic acid, stearic acid and oleic acid) as acyl donors. In a 100ml round-bottom flasks, glucose and fatty acid at different molar proportions (1:0.5 to 1:5) were dissolved in 5ml of organic solvent (acetone, ethyl acetate, tert-butanol, hexane and acetonitrile) to which (0.5-4 ml) of distilled water was added. Reaction mixtures were incubated at different temperatures (25-60 °C) under constant magnetic stirring (400 rpm). The reaction was started by addition of a fixed amount of immobilized lipase beads having different activities (1.2 - 2.7 IU). The time course of the acylation was monitored by estimating residual fatty acid in reaction medium.

2.5. Sugar ester separation and purification

At the end of the reaction, mixture was filtered to remove the enzyme. A volume of (40 ml) distilled water were poured into the filtrate. The product was extracted with 20 ml chloroform. The organic phase was removed in a rotary evaporator. The remaining solid was redissolved in 10 ml of chloroform (or dichloromethane) for further purification step. Glucose ester and fatty acid were separated by silica gel chromatography (**Gilles *et al*, 2012**) as follows: Silica gel G60 (30 g), previously packaged in 70 ml of chloroform, are deposited, reaching a height of 20 cm in a glass column of 2.0 cm diameter. 60 ml of chloroform are eluted before the deposition of the sample. The product (dissolved in 2 ml of chloroform) was placed in the column heading. Elution was successively performed with chloroform (70 ml), a mixture of chloroform / methanol (55:5, 60 ml) and finally with a ternary mixture of chloroform / methanol / water (54/5/1, 60 ml). Solvents were removed in a rotary evaporator and the residual solid was dried and weighed for further analysis.

2.6. Analyses

2.6.1. Acylation yield

The progress of the acylation reaction was followed by determination of fatty acid conversion which was estimated by titrimetric method with sodium hydroxide (0.01 M). Phenolphthalein was used as color indicator. All experiments were performed in duplicate. Blank tests were also conducted under the same conditions in the absence of enzyme. Acylation yield was calculated as the percentage of fatty acid conversion as follows:

Conversion [%] = $[(n_t - ne) / n_0] \times 100$.

with

n_t : fatty acid in blank test (mmol).

n_e : residual fatty acid in test (mmol).

n_0 : initial amount of fatty acid (mmol).

2.6.2. Characterization of glucose ester

The purified product was analyzed by infrared spectroscopy (FT-IR) in the range of 4000-400cm^{-1}. Spectra were compared to those of palmitic acid and glucose.

^{1}H ^{13}C- NMR spectra of the purified product were recorded on a Bruker Avance 300 MHz spectrometer, using samples dissolved in deuterated chloroform.

2.6.3. Surface tension and CMC

The surface activity properties of the product were determined in terms of surface tension which was measured using a Krüss tensiometer (Krüss GmbH, Germany) equipped with a 1.9 cm Du Nouy platinum ring.

The critical micelle concentration (CMC) is a characteristic for a given surfactant in a specific solvent at a defined temperature. The product was dissolved in distilled water, and the surface tension of the solution was measured at various product concentrations under ambient temperature. CMC was measured from the breakpoint of surface tension versus product concentration. Results and discussion

3. Results and discussion

3.1. Effect of organic solvent

Glucose palmitate synthesis was realized in different solvents which were selected on the basis of **solubility of reactants, non-reactivity, the low denaturating effect towards enzyme and low toxicity.** Five solvents were chosen: acetone, tert-butanol, acetonitrile, ethyl acetate and hexane. Effect of solvent type on acylation yield is presented in **Figure 1.** The highest conversion was obtained in tert-butanol, 10% ± 0.153 after 2 h while no product was detected with hexane. These results may be related to several factors such as the low solubility of sugars in organic solvents. For example, the solubility of glucose in acetone and tert-butanol at 60 °C is respectively 0.36 g / L and 2.3 g / L (**In Sang *et al,* 2007**). In addition, most enzymes are quickly inactivated in highly polar organic solvents which are able to dissolve high concentrations of both sugars and fatty acids (**Ganske and Bornscheuer, 2005**). Tert-butanol seems to be the optimal choice as it presents a moderate polarity (1.66 Debye) when compared to acetone (2.7 Debye) and therefore lipase was less desactivated.

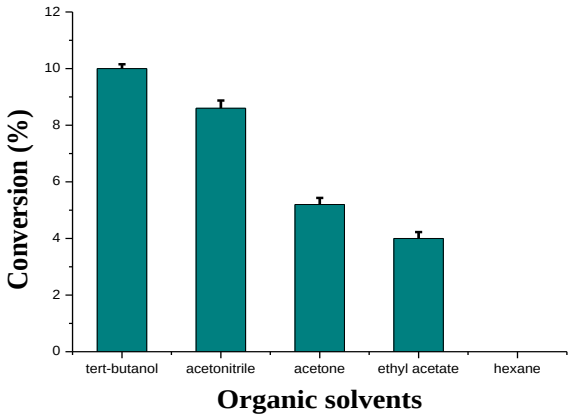

Figure 1. Influence of organic solvents on palmitic acid conversion. Reaction conditions: 1 mmol glucose, 1 mmol palmitic acid, a_w:0.1, 400 rpm and 1.8 IU immobilized lipase in 5 ml organic solvent.

3.2. Nature of acyl donor

Three fatty acids (palmitic acid, oleic acid and stearic acid) were used at a fixed molar ratio of fatty acid to glucose of 1:1 dissolved (5ml) in tert-butanol at room temperature. The Influence of fatty acid type as acyl donors on acylation yield is shown in **Figure 2**. Conversion yields of 10%, 5% and 3% were respectively obtained with palmitic acid, stearic acid and oleic acid after 2 hours of reaction time. Increasing reaction time will increase the conversion yield which reached 26.6% with palmitic acid after 4 hours. **Stereoselectivity of lipases, defined by the selectivity observed depending on the length of the carbon chain is often related to the topology of the active site** (**Jaeger *et al,* 1999**). *Ricinus communis* lipase seems to have specificity toward relatively short saturated fatty acids such as

palmitic acid (16:0) rather than unsaturated with longer chain such as oleic acid (18:1). Palmitic and stearic acids were found to be good acyl donors for sucroesters syntesis in acetone due to the specificity of Novozym 435 **(Thanongsak Ch.,2007)**. Thus, palmitic acid was chosen as the fatty acid to be used for the following study.

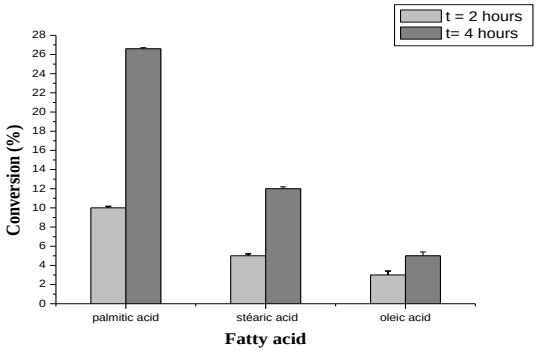

Figure 2. Influence of nature of donor acyl on the conversion.Reaction conditions: 1 mmol glucose, 1 mmol palmitic acid, 1.8 IU immobilized lipase, a_w:0.1, 400 rpm, in 5 ml tert-butanol, room temperature

3.3. Water activity

Eight experiments were performed without and with adding different proportions of distilled water. Conversion of fatty acid (palmitic acid) is significantly affected by the presence of water (**Figure 3**). From data on figure 3, conversion yield was doubled from 10% to 20% ± 1 when water activity reached 30% after 2 hours. These results could be explained by two effects related to water effect on the polarity of the active site of the enzyme and the thermodynamic equilibrium of the reaction. Water molecules are involved in multiple hydrogen bonds and provide conformational mobility which are necessary for the enzyme. A low amount of water would lead to a stiffening of the enzyme which induces inactivity. In contrast to this, water in excess would result in an accumulation of water molecules surrounding the enzyme, thus, forming a diffusion barrier for the hydrophobic substrate and inducing a significant hydrolytic activity. Alternatively, with higher water content, the equilibrium of the reaction is shifted to ester hydrolysis and therefore lower acylation yield. In early studies of biocatalysis in organic solvents, it was observed that the catalytic activity of enzymes correlated well with the amount of water bound to the enzyme (**Adlercreutz, 2013**). Thanongsak et al. found the highest yield of ester formed from glucose and PFAD (94.1% palmitic acid) by *C. Antarctica* lipase was obtained with an initial a_w = 0.07 in acetone (**Thanongsak *et al*, 2006**). Glucose caproate was obtained at a yield of 80.20 % with a_w = 0.33 at 45 °C after 56 h reaction time catalyzed by immobilized lipase from *Streptomyces thermocarboxydus* ME168 (**Thanongsak**, **2007)**.

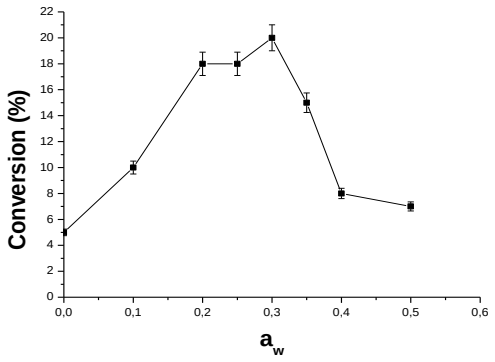

Figure 3. Effect of water activity on the conversion of palmitic acid. Reaction conditions: 1 mmol glucose, 1 mmol palmitic acid, 1.8 IU immobilized lipase, 400 rpm in 5 ml tert-butanol at temperature room.

3.4. Effect of molar ratio $n_{glucose}/n_{palmitic\ acid}$

The influence of molar ratios of glucose to palmitic acid, ranging from 1:0.5 to 1:5, on glucose palmitate production was investigated. Results are shown in **Figure 4**. The molar ratio of the two substrates significantly influenced the conversion yield which increased from 12 to 22.2% when substrate molar ratio was increased from 1:0.5 to 1:2 after 2 hrs. Further increases in molar ratio (above 1:2) had no positive effect on glucose palmitate production, which may be due to the inhibitory effect of high acid concentration on enzyme activity (**Güvenc *et al*, 2002**). Glucose solubility in the reaction medium could be also affected by the concentration of acid by varying the polarity of the reaction medium. Yan et al reported that in reaction of glucose with a saturated long chain fatty acid an excess of the fatty acid in the reaction mixture significantly increased the yield of the sugar ester (**Yan *et al*, 2001**). The same effect was also observed during oleyl oleate production by using Novozym 435 as a biocatalyst, in a solvent-free system (**Güvenc *et al*, 2002; Kapucu *et al*, 2002**). Hence, substrate molar ratio of 1:2 was optimal for glucose palmitate production with the highest conversion yield of 22.2% ± 0.575, and was kept constant during the following tests.

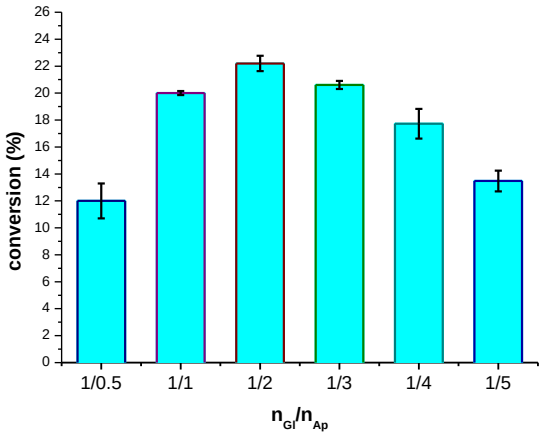

Figure 4. Effect of molar ratio (glucose to palmitic acid) the conversion of palmitic acid. Reaction conditions: 1.8 IU immobilized lipase, a_w: 0.3 , 400 rpm in 5 ml tert-butanol.

3.5. Effect of temperature

To study the influence of temperature on immobilized lipase activity, esterification reactions were performed at temperatures varying from 25 °C to 60 °C in a water bath. The Influence of temperature on acylation yield is shown in **Figure 5**. The conversion was significantly affected by temperature. A maximum conversion rate of 31.6% ± 0.56 was obtained at 40 °C after 2hrs of reaction time.

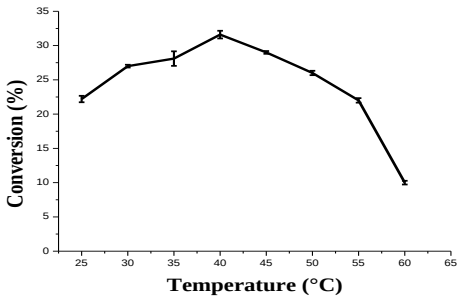

Figure 5. Effect of temperature on the conversion of palmitic acid. Reaction conditions: 1.8 U immobilized lipase, a_w: 0.3, 2 h, 400 rpm, molar ratio (1:2) in 5 ml tert-butanol.

A similar result was obtained using lipase from *Candida sp* (**Zhao *et al.* 2013**). Temperature affects both enzyme activity, the solubility of the reactants and equilibrium. From data shown on **Figure 5,** the conversion of palmitic acid increased in a temperature range from 25 to 40 °C, as enzyme activity and reactants solubility were positively enhanced. However, above 40 °C, the enzyme will become partially inactivated in the organic solvent leading to lower conversion of palimitic acid. For enzymatic synthesis of glucose ester, Yu et al reported an increase in the enzymatic activity as the reaction temperature was raised from 35 to 45 °C (**Yu *et al,* 2008).** Tarahomjoo S. obtained the same result when glucose palmitate was synthesized with lipase from *Candida antarctica* (Novozyme 435) in acetone (**Tarahomjoo S. et *al,* 2003**).

3.6. Effect of immobilized lipase activity

From **figure 6**, immobilized lipase activity increased with enzyme loading. Maximal activity of 2.7 IU was obtained when lipase mass was above 1.4 g. Further increase of enzyme load (up to 2 g) did not affect immobilized lipase activity.

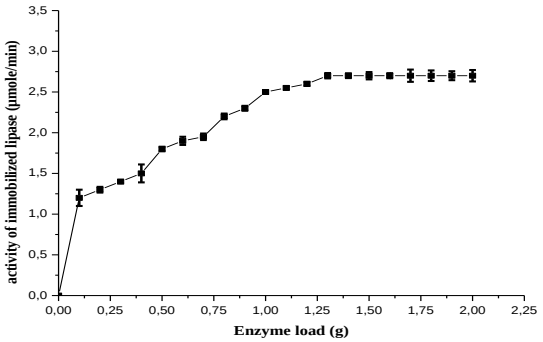

Figure. immobilized lipase activity vs. enzyme loading.

The Influence of immobilized lipase activity on acylation yield was studied. Enzyme activity was varied from 1.2 to 2.7 IU. The effect of lipase activity on acylation yield is illustrated in **figure 7**. The conversion palmitic acid increased with lipase activity till a maximum conversion yield (65% ± 1.01) was obtained at 2.7 IU. Similar behavior was reported for the esterification of glucose by palm fatty acid distillates (PFAD) catalyzed by Novozym 435 indicated, that further increase in enzyme concentration more than 150 IU/ml acetone did not significantly increase the product yield (**Thanongsak C., 2007**).

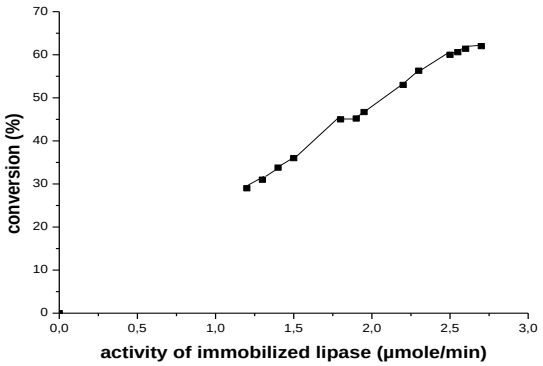

Figure 7. Effect of immobilized lipase activity on the conversion of palmitic acid. Reaction conditions: a_w: 0.3, 8 h, 40 °C, 400 rpm, molar ratio (1:2) in 5 ml tert-butanol.

3.7. Kinetics of glucose palmitate synthesis under the optimal conditions

A kinetic study of glucose palmitate synthesis catalyzed by immobilized *Ricinus communis* lipase was conducted under optimal conditions as follows: solvent (tert-butanol) , water activity (0.3), glucose to palmitate molar ratio (1:2), temperature (40 ° C), and activity of immobilized lipase (2.7 IU). Reaction mixture was kept under constant magnetic agitation 400 rpm. Results are shown on **Figure 8**. Under these conditions, the conversion yield of palmitic acid to glucose ester increased with contact time. A maximal yield of 65% was obtained after 8 h**.** No significant increase was noticed after 8h. Comparable results (89% after 6 h) were obtained when glucose palmitate was synthetised in the presence of lipase of *Candida* sp. 99-125 immobilized on nylon (**Zhao L. *et al*, 2013**). Fructose palmitate was also obtained with a comparable conversion yield (66 %) under 40 °C but after 56 h with *Candida antarctica* lipase B immobilized on a macroporous acrylic resin (Novozyme 435) (**Keiji S. *et al*., 2006**).

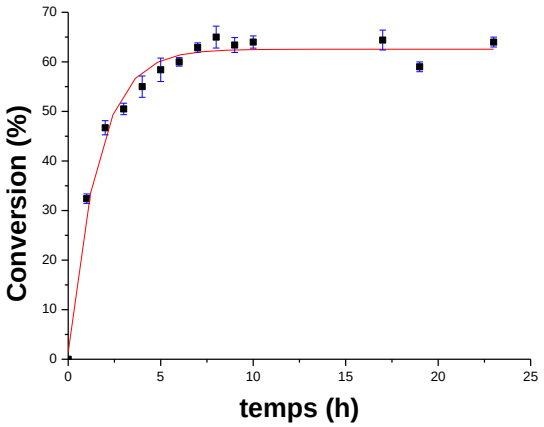

Figure 8. Kinetics of glucose palmitate synthesis under the optimal conditions. 2.7 U immobilized lipase, a_w:0. , 40 °C, 400 rpm, molar ratio (1:2) in 5ml tert-butanol.

3.8. Repeated batch esterification

The reusability of immobilized lipase was tested by transferring alginate particles successively in a fresh reaction medium after 8 hrs (**Cao L. *et al*., 2000; Schoevaart R. *et al*., 2004).** The final conversion yield was measured at the end of experiment. The results are given in **Figure 9**. The immobilized lipase showed good stability in tert-butanol under optimized conditions. Only 20% decrease in conversion yield was noticed even after 10 reaction cycles. Similar results were reported for the synthesis of glucose palmitate using vinyl palmitate as acyl donor by lipase B from *C.antarctica* _SP435 (**Cao L. *et al*., 1998**).

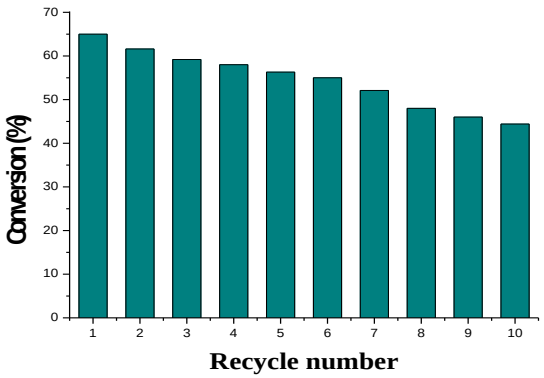

Figure 9. Operational stability of alginate entrapped lipase for glucose palmitate synthesis under optimal conditions

3.9. Structural characterization of glucose palmitate by IR spectroscopy and NMR (^{1}H and ^{13}C)

The FT-IR spectrum for the glucose palmitate is presented in **Figure 12**. Spectra were compared to those of glucose and palmitic acid (**figure 10** and **11** respectively). The band 3421.83 cm^{-1} is due to the hydroxyl stretching vibration. The bands in the region of 2922.25 cm^{-1} and 2850.88 cm^{-1} are due to C-H stretching vibrations of CH_2 groups. While in the region 1745.64 cm^{-1} suggests the presence of carboxyl groups C=O of glucose palmitate. Absorption of CH_3 groups is detected at 1460.16 cm^{-1}. The characteristic absorption at 1166.97 cm^{-1} and 1099.46 cm^{-1} in the FT-IR spectrum was indicative of C-O-C linkage in the glucose palmitate while the absorption at 721.40 cm^{-1} also indicated the presence of linked palmitic acid.

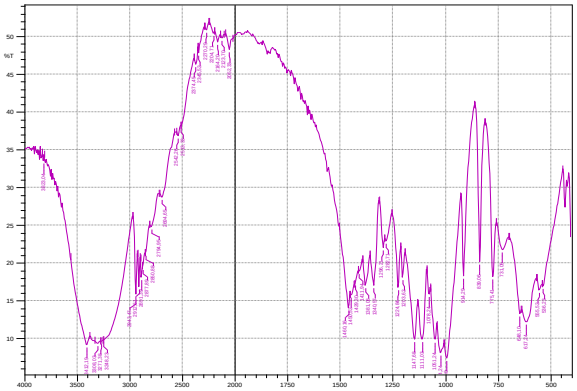

Figure 10. FT-IR spectrum of glucose.

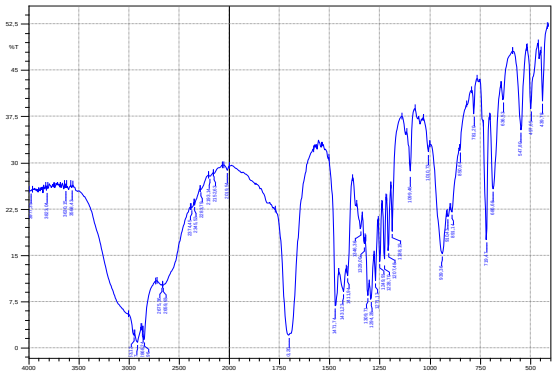

Figure 11. FT-IR spectrum of palmitic acid.

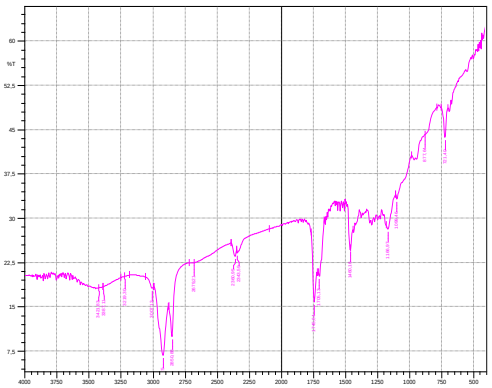

Figure 12. FT-IR spectrum of glucose palmitate synthesized by *Ricinus communis* lipase.

Results obtained from ^{1}H and ^{13}C- NMR indicated that the reaction product has a glucose palmitate structure (**Figure 14)**. **Table.1** gives the $^{1}H$and ^{13}C chemical shifts of NMR signals in the product.

Table.1: ^{1}H and ^{13}C-NMR data of the reaction products in $CDCl_3$.

	^{1}H NMR/ppm	^{13}C NMR/ppm
H(C16)	0.89	14.12
H(C4-C14)	1.29	29.44
H(C15)	1.48	22.66
H(C3)	1.63	24.78
H(C-OH)	2.23	
H(C2)	2.35	34.12
C1		173.10
H(C1'-C6') of glucose	3.64-5.40	133.33-62.11
$CDCl_3$	7.28	76.59-77.44

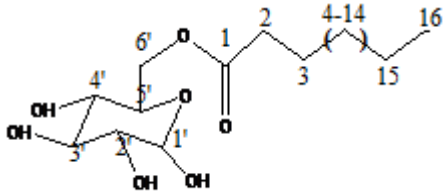

Figure 13. Structure of glucose palmitate

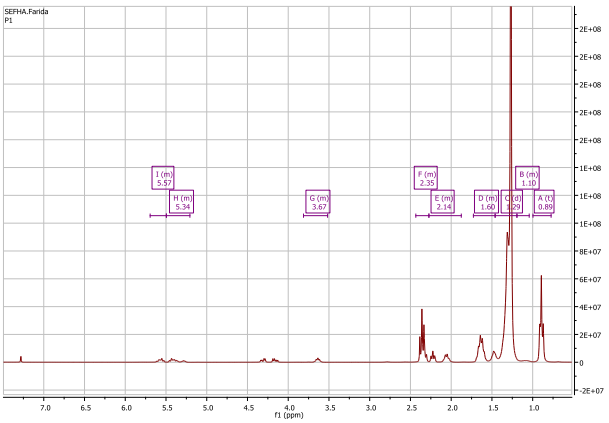

Figure14. ^{1}H-NMR spectrum of glucose palmitate synthesized by *Ricinus communis* lipase.

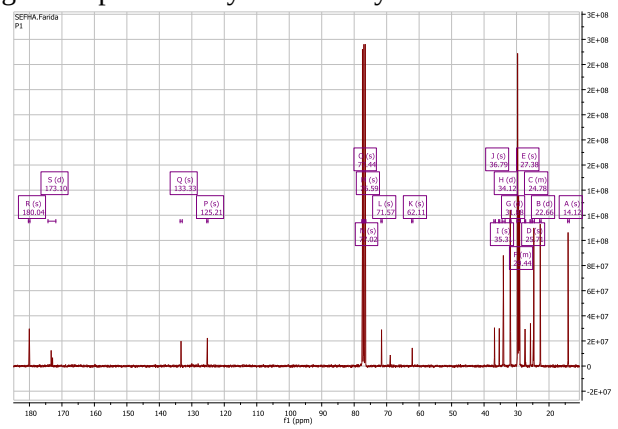

Figure 15. ^{13}C-NMR spectrum of glucose palmitate synthesized by *Ricinus communis* lipase.

3.10. CMC and γ_{CMC}

Figure 16 show the surface tension data for glucose palmitate synthesized by *Ricinus communis* lipase. The obtained results indicated that, the glucose palmitate reduces the surface tension of water from 72.5 to about 41 mN/m. In a certain concentration, any further increase in the concentration of the glucose palmitate was not accompanied by a decrease in surface tension. This concentration corresponds to the point where the glucose palmitate first shows a stable low surface tension value termed CMC. Therefore, CMC and γ_{CMC} were measured from the breakpoint of two lines as 0.1 g/L and 41mN/m respectively. Hakimeh et al. found the lower the surface tension (43 mN/m) obtained with Lipopeptide Biosurfactant from Thermophilic Bacterium Aneurinibacillus thermoaerophilus MK01 Newly Isolated from Municipal Landfill Site (**Sharafi H.et *al*., 2014)**

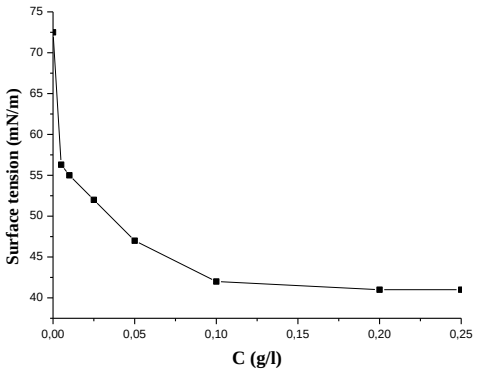

Figure 16. Variation of the surface tension of the glucose palmitate versus concentration

4. Conclusion

Glucose palmitate was successfully synthesized by using *Ricinus communis* immobilized lipase as a catalyst under mild reaction conditions. It was proved that glucose palmitate synthesis was affected by various parameters which were optimized in order to maximize the reaction yield. The highest conversion of glucose and palmitic acid was obtained in tert-butanol (65%), 2.7 IU of lipase, glucose-to-palmitic acid molar ratio of 1:2 and reaction temperature of 40 °C. Under these conditions, the immobilized lipase can be used more than 6 times with less than 15% loss of efficiency. The structure of active groups of purified glucose palmitate was also proved by structural analysis with FTIR, H and ^{13}C-NMR. The CMC value also indicates a promising aspect to use glucose palmitate as a surfactant in food and pharmaceutical industries**.**

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